AD-A233 127

REPORT DOCUMENTATION PAGE

1b. RESTRICTIVE MARKINGS

2a. SECURITY CLASSIFICATION AUTHORITY

3. DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; distribution is unlimited

2b. DECLASSIFICATION/DOWNGRADING SCHEDULE

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
NMRI 90-129

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION
Naval Medical Research Institute

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION
Naval Medical Command

6c. ADDRESS (City, State, and ZIP Code)
8901 Wisconsin Avenue
Bethesda, MD 20889-5055

7b. ADDRESS (City, State, and ZIP Code)
Department of the Navy
Washington, DC 20372-5120

8a. NAME OF FUNDING/SPONSORING ORGANIZATION
Naval Medical Research & Development Command

8b. OFFICE SYMBOL (If applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)
8901 Wisconsin Avenue
Bethesda, MD 20889-5044

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61153N	MR04101.001	1005	DN277001

11. TITLE (Include Security Classification)
Background review and curent concepts of reperfusion injury

12. PERSONAL AUTHOR(S) Hallenbeck JM, Dutka AJ

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM _____ TO _____	1990	10

16. SUPPLEMENTARY NOTATION
Reprinted from: Archives of Neurology 1990 November, Vol.47 No.11 pp. 1245-54

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Stroke, cerebral ischemia, free radicals, white cells, reperfusion injury

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL
Phyllis Blum, Librarian.

22b. TELEPHONE (Include Area Code)
(301) 295-2188

22c. OFFICE SYMBOL
MRL/NMRI

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

Reprinted from the Archives of Neurology
November 1990, Volume 47

Background Review and Current Concepts of Reperfusion Injury

John M. Hallenbeck, MD, Andrew J. Dutka, MD

• **We define the concept of reperfusion injury, and we present a background chronology of experimental work supporting and questioning this concept. We identify several new influences, such as current clinical interest in thrombolytic therapy for acute ischemia of heart and brain and the growing recognition of endothelium as a regulator of homeostasis. We propose that these influences will encourage a reexamination of reperfusion injury as a factor in the ultimate outcome of tissue exposed to reversible ischemia. We briefly discuss the major mechanisms presently implicated in reperfusion injury—loss of calcium homeostasis, free radical generation, leukocyte-mediated injury, and acute hypercholesterolemia.**

(*Arch Neurol.* 1990;47:1245-1254)

During focal ischemia, the extent of parenchymal tissue damage relates closely to two factors: the level of blood flow during the ischemic interval and the duration of ischemia (Fig 1, top).[1,2]

On return of blood flow, there is a resumption of the principal functions of tissue perfusion: oxygen delivery, provision of substrates for metabolism, and clearance of metabolic wastes. The restored perfusion counteracts the ischemic injury process and serves to return at least some of the reversibly injured tissue to a functional state. However, there is a developing consensus that return of blood flow in the postischemic period has a dark side.[3,4] Interactions between blood and the damaged tissue can lead to further tissue injury. This theoretical construct implies that unmodified blood is not an ideal perfusate for reflow to tissue subjected to prior ischemia. Its reintroduction can actually contribute to parenchymal cell loss as an unwanted side effect of its more familiar capacity to salvage reversibly damaged cells. This paradoxically harmful aspect of blood flow return has been termed *reperfusion injury* (Fig 1, bottom), and the process has potential relevance to clinical medicine. Although reperfusion per se is essential to functional recovery and reduces the volume of tissue infarcted, a further reduction in tissue loss might be achieved by measures that counteract reperfusion injury. The present review addresses the major mechanisms currently implicated in reperfusion injury as observed in brain, heart, and other organs. The role of excitotoxins is not emphasized in this review.

Accepted for publication April 30, 1990.

From the Neurology Department, National Naval Medical Center, Bethesda, Md.

The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

Reprint requests to Neurology Department, National Naval Medical Center, 8901 Wisconsin Ave, Bethesda, MD 20814-5011 (Dr Hallenbeck).

BACKGROUND

Reperfusion injury occurs in a variety of tissues, but much of the work on these phenomena has been done in heart and brain. Concepts of reperfusion injury tend to emphasize similarities between mechanisms of cell death in various tissues rather than properties unique to a given tissue. Concern about myocardial reperfusion was expressed by Jennings et al[5] in 1960, when they suggested that reperfusion may accelerate the development of necrosis in irreversibly injured myocytes. They observed an ultrastructural appearance of "explosive swelling," which included architectural disruption, contraction bands, and intramitochondrial calcium phosphate granules. In 1977, Bulkley and Hutchins[6] reported the paradox of myocardial necrosis after successful revascularization by coronary artery bypass graft surgery and suggested that the lesions were operation related and represented contracture due to calcium loading and myocardial cellular edema in the distribution of widely patent arterial grafts. They further concluded that "prevention of intraoperative myocardial injury must also focus on characteristics of the phase of myocardial reperfusion." Studies by Greene and Weisfeldt[7] and by Shine and Douglas[8] demonstrated that measures instituted after the termination of ischemia, which attenuated the rise in myocardial cytosolic calcium, led to a reduction of tissue injury. Subsequently, other workers have implicated cell swelling,[9] white blood cell plugging of vessels,[10] and free radical damage[11] in reperfusion injury of the heart.

Ames and coworkers[12] focused attention on the contribution of reperfusion impairment to neural injury. Based on a disproportionately long survival of retinal ganglion cells exposed to anoxia in culture (in which oxygen could diffuse directly to the cells when reintroduced rather than requiring an intact tissue perfusion) as compared with the rapidity with which ischemia in vivo leads to irreversible brain cell damage,[13] they postulated that recirculation of ischemic brain could become compromised and account for the differences in neuronal vulnerability observed under the two conditions. This impairment of microvascular reperfusion was attributed primarily to two phenomena. The first was narrowing of the capillary lumen due to perivascular swelling and formation of endothelial blebs, and the second was increased blood viscosity. Fluid shifts into cells, with their sodium-potassium adenosine triphosphatase pumps shut down by energy failure, were thought to underlie both the capillary narrowing and the microvascular hemoconcentration with increased blood viscosity. This same

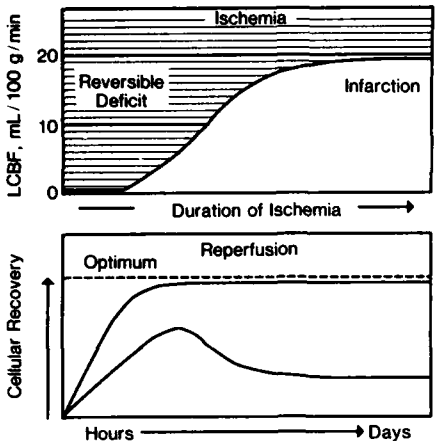

Fig 1.—Top, Interaction between the level of residual blood flow and the duration of ischemia in an ischemic injury zone that determines the amount and degree of tissue damage. During ischemia, lower residual flows are tolerated for shorter periods than higher residual flows. LCBF indicates local cerebral blood flow. Bottom, The upper curve represents a theoretically optimal reperfusion devoid of any injurious influence on the previously ischemic tissue. The return of oxygen and substrates for metabolism and the clearance of metabolic wastes promote a rapid recovery of cellular function that reaches the maximum possible level and is sustained. In contrast, the lower curve denotes reperfusion injury with consequent retardation and attenuation of cellular recovery. Furthermore, the maximal level of recovery is not sustained, but yields instead to progressive secondary damage.

laboratory adduced experimental support for these concepts in a series of articles.[14] In addition, others described the topography of "no reflow,"[15] and published provocative studies have demonstrated the resumption of function in cortical neurons after total ischemia for periods of 30 minutes to 1 hour if measures were taken to overcome reperfusion impairment.[16,17]

The theory that vessel shutdown during ischemia could occur at a time when nerve cells were still potentially viable collided with a deeply ingrained tenet that neurons are uniquely sensitive to hypoxia and ischemia. The no-reflow theory was attacked on the grounds that the observed distribution of microvessel occlusion did not correspond to the distribution of neurons that exhibit selective vulnerability and that compression of the vascular lumen by swollen perivascular astrocytes and swollen endothelial cells with bleb formation was seldom observed in histopathological material.[18] Several experiments in gerbils revealed that extensive postischemic neuronal damage could occur with a patent microvascular bed[19] and that elimination of focal microcirculatory flow deficits by raising blood pressure had no effect on mortality.[20] Some authors developed the view that no reflow was a consequence of ischemic brain injury rather than a proximal cause of it.[21]

The concept of a multifocal impairment of reflow manifesting immediately after ischemia and decreasing with time was modified and extended by consideration of "autodestruction" in models of spinal cord trauma and reperfusion problems encountered during autotransplantation of organs preserved by ex vivo perfusion.[22] Returning blood flow was viewed as having two dichotomous effects: (1) a well-established restorative effect and (2) a postulated capacity to undergo a multifactoral interaction with damage tissue that could progressively shut down microcirculatory flows and contribute to further neuronal damage. Levy et al[23] measured blood flow and metabolism in gerbils subjected to unilateral carotid occlusion, and they noted an immediate return of perfusion to the brain on release of the occlusion, followed in 10 to 30 minutes by a decline in flow for at least 4 hours with a coincident rise in the cerebral metabolic rate that disrupted the normal flow-metabolism couple. They introduced the term *delayed postischemic hypoperfusion* to describe this phenomenon. Pulsinelli and colleagues[24] then documented this phenomenon in the four-vessel occlusion model that they had devised in rats.

In 1981, Siesjo[25] published his speculative synthesis, which culminated with the insight that elevation of the cytosolic calcium level may be a pivotal factor that leads to cell death in such diverse conditions as ischemia, hypoglycemia, and status epilepticus. The disruption of calcium homeostasis was attributed to energy failure in ischemia and hypoglycemia and to overloading of calcium transport and sequestration mechanisms in status epilepticus. Among many other studies, a provocative set of results reported by Schanne et al[26] in 1979 was cited in support of this hypothesis. Schanne and associates[26] had observed that the expression of toxicity of a variety of membrane toxins, added to cultured hepatocytes, depended on the presence of calcium in the culture medium. That is, calcium was required for the death of cells in that system. The clear and compelling reasoning in the communication of Siesjo[25] focused attention on mechanisms for continuing cell damage that were intrinsic to the neuron and tended not to emphasize events at the blood-endothelial interface. Further interest in neuronal self-destruction has been fostered by the observation in a number of models that a process of delayed neuronal death can progress for days after brief ischemia[27,28] and may involve excitotoxins, such as glutamate and aspartate acting via the *N*-methyl-D-aspartate[29] receptor to initiate an accumulation of calcium ion in the cytosol via agonist-operated channels. Because the transition of glutamate from neurotransmitter to neurotoxin may depend on elimination of the voltage-dependent Mg^{2+} block of the *N*-methyl-D-aspartate receptor channel by local interference with glucose and oxygen delivery as shown in cultured cerebellar neurons,[30] progressive microcirculatory flow shutdown could act in concert with excitotoxins to produce delayed neuronal death. That is, these two potential mechanisms for delayed neuronal death may not be mutually exclusive.

In recent years, several new developments have returned attention to blood elements, blood vessels, and blood flow in ischemically damaged tissue. The demonstration that coronary thrombi were obstructing vessels that supplied ischemic myocardium in the great majority of patients studied angiographically during the first hours of acute transmural myocardial infarction[31] and experiments that indicated that myocardium can be salvaged by reperfusion led to efforts to lyse the offending thrombi and reopen supply vessels.[32,33] Several trials of thrombolysis in cerebrovascular disease are also under way.[34] These initiatives bring to the fore the problem of reperfusion injury in a clinical setting.

Another force that impels consideration of blood elements, blood vessels, and blood flow during and after ischemia derives from work in endothelial cell tissue culture. Results of this work implicate endothelium as a regulator of hemostasis. Endothelium is not simply a passive, nonthrombogenic surface that separates blood from tissue.[35,36] It is endowed with ultrastructural specializations, such as projections, caveolae, and microdomains. Indeed, endothelial projections or microvilli have been observed to proliferate after ischemia, indicating membrane activation and reducing to some degree the vascular lumen.[37] There is a carpet of molecules on the luminal surface of endothelium called the glycocalyx that has not been fully characterized. It is a well-hydrated, complex, cross-linked polymer that contains fibronectin, a form of collagen, glycosaminoglycans, and probably fibrin

and fibrinogen. The apparent functions of the glycocalyx include regulating and restricting access to surface receptors and enzymes and, also, molecular sieving.

Under normal circumstances, the endothelium presents to the blood an actively anticoagulant and antithrombotic surface. The thrombomodulin-protein C-protein S system is a major contributor to this anticoagulant property.[38,39] Thrombomodulin on the endothelial surface binds thrombin and alters that molecule so that fibrinogen is no longer a substrate, and instead, the thrombomodulin-thrombin complex activates protein C. Protein C is bound to the endothelial cell surface and also to the surface of other cell membranes by protein S and in the bound state will inactivate factors V and VIII and will also inactivate tissue plasminogen activator (TPA) inhibitor. Tissue plasminogen activator is also released from the endothelium.[40] It is a serine-centered protease of about 68 to 70 kd, and it activates plasminogen by cleavage of a single arginine-valine bond. It converts very little plasminogen to plasmin in the absence of fibrin, but when fibrin is present, its activity increases by two to three orders of magnitude. This enzyme therefore has a predilection for lysis of local clot rather than circulating fibrinogen.[41] Heparinlike molecules have been demonstrated on the endothelial surface by iodine 125-antithrombin III binding studies.[42] Prostacyclin is produced in response to various forms of membrane perturbation and is a potent vasodilator and the most potent inhibitor of platelet aggregation known.[43] Adenosine diphosphatase is present on endothelium localized to caveolae on the luminal surface.[35] This enzyme, acting in conjunction with 5′-nucleotidase, converts adenosine diphosphate to adenosine monophosphate to adenosine.[44] The adenosine diphosphate is a potent mediator of platelet aggregation, but after conversion to adenosine, it mediates platelet inhibition and vasodilatation. There is also a general protease inhibitor, α_2-macroglobulin, on the endothelial surface.[45]

Under the influence of a variety of mediators, endothelium can undergo a "Jekyll to Hyde" transformation and become actively procoagulant. This can occur with morphologically imperceptible endothelial cell injury.[46] It used to be thought that endothelial cell denudation was necessary to make vessels thrombogenic. This concept was due in part to the use of large-vessel models in which endothelium was denuded for the study of platelet adhesion and aggregation and, also, to in vitro collagen-induced platelet aggregation studies, suggesting the need for subendothelial collagen (said actually to be of a nonthrombogenic type).

The procoagulant property of endothelium involves many facets. Tissue factor expression, which initiates the extrinsic pathway of coagulation, is stimulated by thrombin, interleukin 1 (IL-1), tumor necrosis factor (TNF), and other stimuli.[47,48] Interferon gamma or the presence of other cells, such as platelets, leukocytes, lymphocytes, and probably also monocytes, greatly increases the expression of tissue factor in response to any given stimulus.[49] Such expression requires RNA and protein synthesis.

Endothelial cells are both a source and a target for IL-1.[50] Synthesis of this cytokine is stimulated by TNF and endotoxin. Interleukin 1 induces expression of endothelial cell procoagulant activity (tissue factor),[48] formation of platelet activating factor (PAF),[51] and inhibition of protein S membrane binding interfering with the thrombomodulin-protein C-protein S system,[52] and it also induces expression of surface receptors for leukocyte binding,[53] the endothelium-leukocyte adhesion molecules.[54] Another leukocyte adhesion molecule, intercellular adhesion molecule-1, is expressed on endothelial cells on stimulation with TNF and IL-1.[55] Endothelium-leukocyte adhesion molecule and intercellular adhesion molecule-1 receptors require 4 to 6 hours for maximal expression. Rapid binding of neutrophils to endothelial cells appears to involve the type 3 complement receptor, CR3, and iC3b, the only known complement factor that binds to CR3.[56] Peak adhesion mediated by this receptor occurs within 20 minutes of activation. Endothelial cells also produce a neutrophil chemotactic factor (molecular weight, 7.5 kd) in response to stimulation by TNF or IL-1[57] and can release a factor that activates the Hageman factor.[58] Factor VIII/von Willebrand factor is released from endothelial cells at a basal rate related to protein synthesis and also through a calcium-dependent mechanism from storage sites[59] in response to thrombin and other mediators.[60]

Thromboxane A_2, a potent vasoconstrictor and platelet aggregator, is produced by endothelial cells in a ratio of about one fifth to one tenth that of prostacyclin.[61] This ratio may change in pathological states. There is a 5-lipoxygenase system present in vessels that enables them to produce leukotrienes with appropriate stimulation.[62] Among the multiple effects of leukotrienes are chemotaxis for various leukocytes, vasoconstriction, and increased vascular permeability.[63]

Endothelial cells produce PAF in response to IL-1, thrombin, and a number of phlogistic mediators.[64] Prostacyclin that tends to be concurrently released feeds back and inhibits the release, to some extent, of PAF.[65,66] The effects of PAF include platelet accumulation and aggregation, accumulation and activation of leukocytes, increased vascular permeability, vasoconstriction, activation of macrophages to produce IL-1,[64] and modulation of TNF production by activated macrophages.[67]

Endothelial cells provide a physiologically important surface for the activation of prothrombin.[68] They bind and promote activation of factors IX and X in the absence of platelets and exogenous lipid.[69] Each cell can bind on the order of 9 million molecules of factor X. They also synthesize, express, and release factor V.[70]

A rapid inhibitor of TPA with a molecular weight of about 50 kd is produced by endothelial cells.[71] The concentration of this protease is normally less than 10 ng/mL, but baseline concentrations can increase 50-fold or more in severely ill patients or after surgery.[72] It is interesting that TPA and TPA inhibitor concentrations in blood tend to undergo a diurnal variation, with a relatively prothrombotic state (a low TPA and a high TPA inhibitor) occurring early in the morning.[73]

Recent evidence indicates that endothelial cells produce superoxide under certain circumstances.[74] This may have a major role in reperfusion injury.

Vessel tone is influenced by endothelial cells as recently elucidated by Furchgott and Zawadzki.[75] Acetylcholine dilates intact arteries, but tends to cause vasoconstriction when the vessels have been stripped of endothelium. This has led to the concept of an endothelium-derived relaxing factor, which preliminary evidence suggests may be nitric oxide.[76] The endothelium-derived relaxing factor response to acetylcholine is lost in cerebral microvessels after mild, sublethal injury in which the endothelial cells remain morphologically normal. It is a potentially important principle that constrictors of vessels, such as norepinephrine and serotonin, release endothelium-derived relaxing factor on binding to their receptors, and the interplay of the two mediators deter-

mines the amount of vasoconstriction.[46] With endothelial cell damage, the unopposed mediators of vessel contraction lead to enhanced vasoconstriction and, potentially, a reduction of blood flow to critical levels. In addition to its influence on vasomotor tone, endothelium-derived relaxing factor has also been shown to inhibit adhesion of platelets to endothelium.[77]

A 21-residue vasoconstrictor peptide has been isolated from endothelium, sequenced, and designated *endothelin*.[78] It is the most potent mammalian vasoconstrictor known to date.

Thus, the intricate and interwoven mechanisms that are being elucidated in endothelial cell tissue culture confer on the blood-endothelial interface the capacity for a variety of cell-damaging reactions during ischemia and reperfusion. Such studies will encourage a reexamination of local blood and vessel wall interactions during the early return of blood flow to previously ischemic tissue.

MAJOR POSTULATED MEDIATORS OF REPERFUSION INJURY

Calcium

Calcium has been implicated in reperfusion injury under several circumstances. Zimmerman and Hulsmann[79] described a "calcium paradox" in which hearts perfused with calcium-free media for a brief period developed sarcolemmal damage, such that reintroduction of calcium caused massive intracellular influx of this ion with consequent tissue disruption, enzyme release, development of contracture, and marked reductions of high-energy phosphate stores. This circumstance is peculiar to situations in which a heart is temporarily exposed to an artificial perfusate that is deficient in calcium. Young[80] has extended this concept to traumatically or ischemically injured brain. Extracellular calcium in these injured areas has been observed to fall from concentrations greater than 1 mmol to less than 0.01 mmol within seconds to minutes as this cation pours into those critically injured cells that have lost their capacity to maintain calcium homeostasis. The calcium concentration in the extracellular space then rises during a period of hours due to calcium diffusion from surrounding tissues. The surviving neurons in the lesion site, which initially maintained their calcium homeostasis, may therefore be exposed to the same stimuli that produce the calcium paradox in cardiac tissues. Delayed acceleration of tissue damage has been observed to occur at about the time extracellular calcium concentration recovers in models of traumatic and ischemic neuronal injury. This delayed neuronal damage has been attributed to a calcium paradoxlike reaction that occurs in injured nervous system tissues on restoration of extracellular calcium levels toward normal.

Calcium homeostasis can also be disrupted by ischemia (Fig 2)[4,25,81-84] due to interference with sodium-potassium adenosine triphosphatase function, which depolarizes cell membranes and permits intracellular sodium accumulation. This can cause opening of voltage-dependent calcium channels and reverse the sodium-calcium antiport system, such that calcium enters the cell as sodium is extruded. The major emphasis in the neuroscience literature is currently on the activation of *N*-methyl-D-aspartate receptors in response to glutamate and other excitotoxins that are released during and after ischemia with a consequent rise of cytosolic calcium. Calcium-activated nonspecific cation channels may also be opened. In addition, sequestering mechanisms within cells become impaired, and calcium moves from the endoplasmic reticulum into the cytosol. The cellular disturbances associated with elevation of cytosolic calcium include activation of proteases and phospholipases (which can lead to free radical generation), membrane damage, and formation of arachidonic acid metabolites. Also, mitochondria can become overloaded with calcium, which impairs production of adenosine triphosphate. Reperfusion can cause an acute acceleration of calcium influx into cells by injuring cell membranes with a consequent increased membrane permeability.[4,83]

The exact role of cytosolic calcium accumulation in the evolution of neuronal and myocardial injury after ischemia remains to be elucidated, but there is considerable evidence that ischemia and reperfusion are associated with a severe disruption of calcium homeostasis. Recent reports demonstrate, however, that considerable functional impairment can occur in the absence of calcium entry into cells[85] and that the concentration of intracellular calcium can rise considerably without leading to permanent damage.[86] Moreover, the results of calcium channel blocker treatment trials have been mixed in a variety of models.[87,88] The contribution of intracellular calcium flooding to cell death during ischemia and reperfusion should be viewed not as established fact but rather as an attractive hypothesis that is currently undergoing intensive evaluation.[83,89]

Free Radicals

The following is based on three reviews.[90-92] A free radical is a molecule or molecular fragment that contains one or more unpaired electrons in its outer orbital and is, in effect, an open bond. The presence of an unpaired single electron in the outer orbital of a free radical is conventionally represented by a superscript dot ($R\dot{V}$). Molecular oxygen is a biradical with an unpaired electron in each of its two outer orbitals, both spinning in the same direction (ie, parallel), which reduces the reactivity of this biradical because its divalent reduction by direct addition of a pair of electrons is restricted. Most of the electron donors available to oxygen will have electrons with antiparallel spins that occupy the valence orbitals, as this is a lower energy condition. For molecular oxygen to remove two electrons concomitantly from a nonradical molecule that has the normal outer orbital configuration of pairs of electrons spinning in opposite directions, an electron spin inversion must first occur during the association of oxygen with the reducing agent.

Because the spin inversion process is slow ($\simeq 10^{-8}$ seconds) compared with the lifetime of collisional complexes ($\simeq 10^{-15}$ seconds), oxygen in its ground state is a relatively weak oxidant. The restriction in the oxidizing capabilities of molecular oxygen is removed when it acquires one electron at a time because under these circumstances, spin inversion need not occur. This is the univalent pathway of oxygen reduction (Fig 3), and sequential addition of one electron after another to molecular oxygen produces first the superoxide anion radical ($O_2^{\cdot-}$), then hydrogen peroxide (H_2O_2), and then the hydroxyl radical ($OH^{\cdot}$), followed by water as the final product.

The reactivity of molecular oxygen can be increased by reactions that invert the spin of one of the electrons in its two outer orbitals. The UV spectra of such oxygen molecules contain singlet lines that indicate the presence of electrons with antiparallel spins in the outer orbitals. These molecules, termed *singlet oxygen*, are highly reactive because of the removal of quantum mechanical spin restriction so that they can undergo divalent reduction. There are two types of singlet oxygen, a short-lived sigma singlet oxygen ($^1\Sigma g^+$) in which the electrons occupy separate orbitals and a a longer-lived delta singlet oxygen ($^1\Delta gO_2$) in

which the two electrons occupy the same orbital and have opposite spin directions. Singlet oxygen can be formed by light, acting on molecular oxygen in the presence of a photosensitizer. Other postulated reactions involve dismutation of the superoxide anion radical and the oxidation of halides by hydrogen peroxide, a reaction catalyzed by myeloperoxidase, as occurs during the antimicrobial activity of inflammatory cells. Delta singlet oxygen does not fulfill the definition of a free radical but can rapidly oxidize many molecules, including polyunsaturated fatty acids in cell membranes.

Although a number of enzyme systems appear to be capable of catalyzing the univalent reduction of molecular oxygen to the superoxide anion radical, interest in the reperfusion injury field has been focused primarily on two major systems, the xanthine oxidase system[93] and the nicotinamide-adenine dinucleotide phosphate with the reduced form (NADP[H]), NAD[H] oxidase system (Fig 4).[94] Due to the low levels of xanthine oxidase in brain, the relevance of the xanthine oxidase system to reperfusion injury in that organ remains an open question. Several recent articles suggest that xanthine oxidase is formed in ischemic brain and may generate free radicals and contribute to neuronal injury during postischemic reperfusion.[95,96]

Another potentially important source of the superoxide anion radical during ischemia is a "univalent leak" of this radical species from the mitochondrial electron transport system.

The next step in the univalent reduction of oxygen involves conversion of the superoxide anion radical to H_2O_2. This can occur spontaneously but is greatly accelerated by the enzyme, superoxide dismutase, in the following reaction:

$$O^{-}_2 + O^{-}_2 + 2H^+ \rightarrow H_2O_2 + O_2$$

The $OH^{\cdot}$, the next step in the univalent O_2 reduction pathway, can be formed via two reactions: (1) The "Fenton" reaction,

$$Fe^{2} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$$

and (2) the iron-catalyzed "Haber-Weiss" reaction,

$$O_2^{-} + H_2O_2 \rightarrow Fe^{3+}O_2 + OH^{\cdot} + OH^-.$$

Due to the reactivity of $OH^{\cdot}$, no enzyme systems exist that involve it as a substrate. Instead, all efforts of the cell are directed at avoiding its formation. The superoxide anion radical is scavenged by superoxide dismutase, so that it is not available for the Haber-Weiss reaction and H_2O_2 can be removed by reactions catalyzed by glutathione peroxidase,

$$2\,GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

and catalase,

$$2H_2O_2 \rightarrow O_2 + 2H_2O.$$

If these protective reactions should become insufficient to contain free radicals as they are formed, O^{-}_2 and H_2O_2 can diffuse across cell membranes to the extracellular space in which antioxidant defense mechanisms are relatively sparse. In the presence of trace concentrations of transition metal ions, $OH^{\cdot}$ is formed that can initiate free radical chain reactions in cell membranes, causing peroxidation of polyunsaturated fatty acids. Such peroxidation severely damages the cell membrane and causes loss of membrane fluidity and collapse of transmembrane ionic gradients.

A novel pathway for superoxide generation in response to vessel injury has been elucidated and experimentally supported principally in models of brain trauma with acute hypertension.[97] Such perturbation activates phospholipases in the vessel wall, leading to a release of free arachidonate. The released free arachidonate causes accelerated metabolism via cyclooxygenase, producing PGG_2 (a cyclic endoperoxide) that is then converted to PGH_2 (a cyclic endoperoxide) by the hydroperoxidase reaction. This step generates superoxide as a by-product that escapes to the extracellular space via the anion channel. Subsequent reactions can form H_2O_2 and $OH^{\cdot}$, causing membrane damage to cells of the vessel wall. As with disruption of calcium homeostasis, the importance of oxygen-derived free radical formation in reperfusion injury is a research question under active investigation.

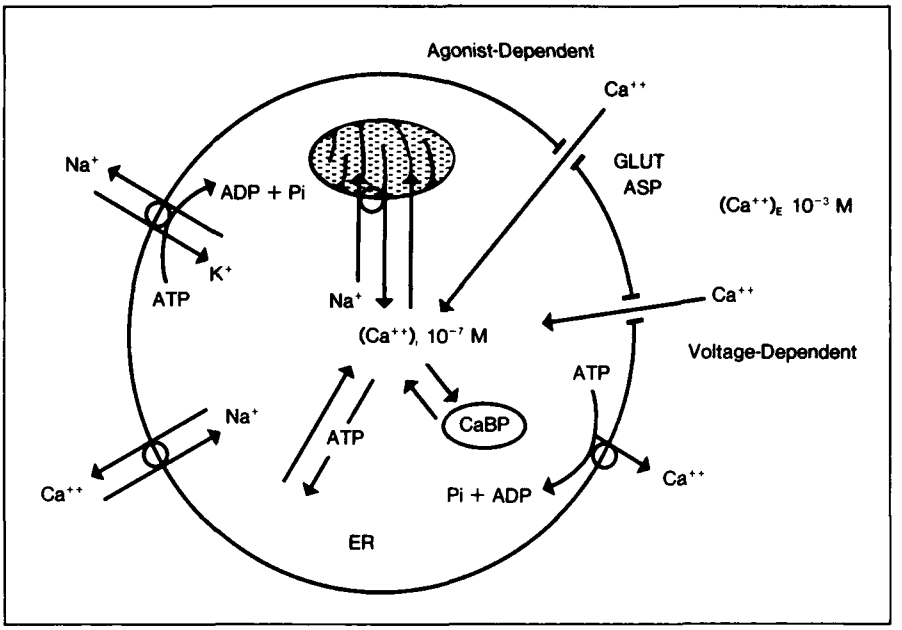

Fig 2.—A 10 000-fold concentration difference between intracellular and extracellular calcium and the internal negativity of the cell create a steep electrochemical gradient for calcium entry, which is normally tightly regulated by voltage- and agonist-dependent channels. Calcium that does enter can be extruded by means of a number of adenosine triphosphate (ATP)-dependent processes, including a calcium-activated ATPase and an electrogenic (3:1) Na^+/Ca^{2+} antiport system driven by the sodium gradient that is itself maintained by the sodium-potassium ATPase. Excess intracellular calcium can be sequestered in endoplasmic reticulum (ER) by an ATP-dependent process and can also associate with a variety of calcium-binding proteins (CaBP). Mitochondria possess a high-capacity uniport mechanism for calcium uptake, which relies on the potential across the inner mitochondrial membrane to translocate electrophoretically calcium internally. Intramitochondrial calcium levels can normally be regulated by an antiport system that exchanges 2 Na^+ for 1 Ca^{2+}. As the capacity of this efflux pathway is less than 10% of the influx pathway, it can be overwhelmed under conditions of markedly elevated levels of free cytosolic calcium. During ischemia, ATP production becomes impaired, and ATP-dependent processes fail. Membrane gradients for sodium and potassium cannot be maintained, and the membrane depolarizes opening voltage-dependent calcium channels. Excitatory neurotransmitters, glutamate (GLUT), and aspartate (ASP), are released and act via the *N*-methyl-D-aspartate receptor to increase further calcium influx. As intracellular sodium rises, the Na^+/Ca^{2+} antiport system becomes reversed and translocates calcium into the cell. Sequestration mechanisms become overwhelmed or stalled by lack of ATP, and mitochondria become flooded with calcium (modified from Siesjo[132]).

Leukocytes

The scope and diversity of possible leukocyte reactions in a zone of tissue injury that may involve rheologic ef-

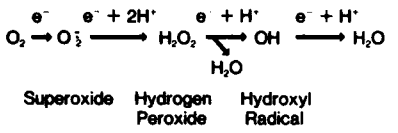

Fig 3.—The univalent pathway of oxygen reduction (where e^- is electron and H^+, hydrogen ion).

fects, toxic oxygen products, granule constituents, and phospholipase products qualify these cells for a central role in many of the postulated modes of reperfusion injury.[98,99] As large, stiff, viscoelastic cells that adhere to endothelium, activated leukocytes can obstruct individual capillaries in an ischemic injury zone and increase diffusion distances for oxygen within the microenvironment of individual nerve cells.[100] Leukocytes can also generate a variety of reduced oxygen products that are capable of initiating lipid peroxidation in cell membranes and damaging key intracellular components.[94] In addition, neutrophil granules contain a number of constituents capable of mediating tissue injury.[98,101] It was initially assumed that extracellular release of cytotoxic products occurred only after the death of neutrophils in inflammatory lesions, but it is now recognized that viable leukocytes that have been activated can release such products. Also, activation of phospholipases in leukocytes can initiate a cascade of reactions that produce such vasoactive substances as leukotrienes and PAF.[98,102] Vasoconstriction, increased permeability, and increased leukocyte adherence are among the vascular effects of these mediators.

There are a variety of endogenous inhibitors and regulators of leukocyte-mediated vascular injury. These include chemotactic factor inhibitor,[103] neutrophil-derived proteases,[104] and neutrophil-generated oxidants[105] that inactivate chemotaxins, and plasma factors that inactivate PAF[106] and neutral proteases.[107] Scavengers[108,109] and antioxidant enzymes,[110,111] which clear neutrophil-generated oxidants, also participate. There is an apparent requirement for neutrophil adhesion before endothelial cell damage by activated neutrophils can proceed.[98] Adhesion under these circumstances seems to create an environment that is protected from endogenous inhibitors and thus permits leukocyte-mediated vascular injury to progress unhindered. This apparent requirement supplies a rationale for therapy directed at inhibiting leukocyte adhesion.

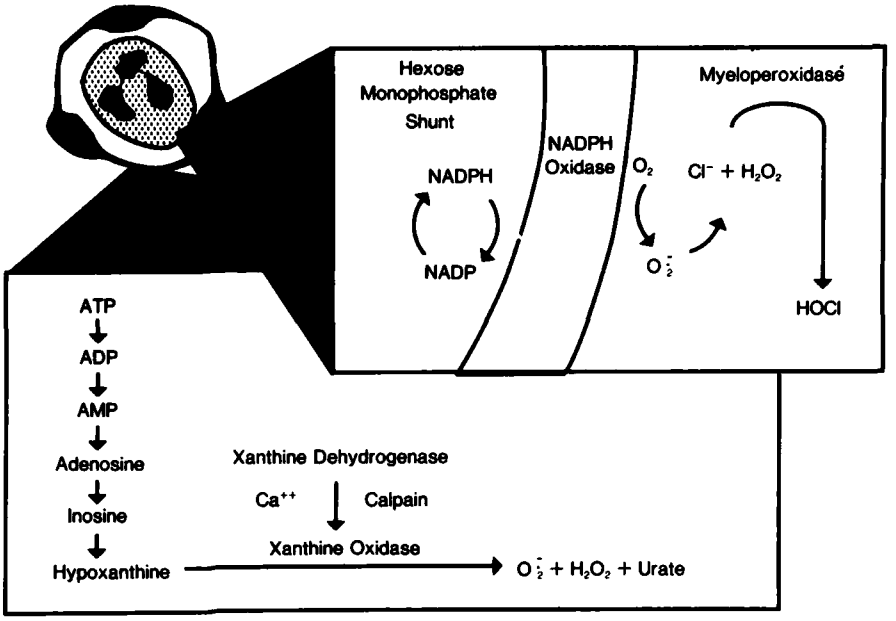

Fig 4.—Two free radical generating systems are depicted. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system in leukocyte membranes (neutrophils, eosinophils, monocytes, and macrophages) has a pyridine nucleotide binding site on the cytoplasmic surface and an O_2^- forming oxidase component in the lipid bilayer so that O_2^- is generated on the external surface of the plasma membrane. Subsequent reactions produce hydrogen peroxide (H_2O_2) that can combine with Cl^- in a reaction catalyzed by myeloperoxidase to produce hypochlorous acid (HOCl). The xanthine oxidase system is concentrated in endothelium. During ischemia, xanthine dehydrogenase is converted by a calcium-activated protease (possibly calpain) to xanthine oxidase, an enzyme that produces O_2^-, H_2O_2, and uric acid from hypoxanthine and molecular oxygen. The hypoxanthine substrate that is supplied by the ischemia-induced degradation of adenine nucleotides and molecular oxygen, which is not shown in the diagram, becomes available during reperfusion. ATP indicates adenosine triphosphate; ADP, adenosine diphosphate; and AMP, adenosine monophosphate.

Relative to the red blood cell, the leukocyte has roughly double the volume, a coefficient of viscosity that is 2100 times greater, and an entry time into capillaries that is three orders of magnitude greater.[99,100] Activated leukocytes undergo receptor-mediated adhesion to intact endothelium by means of the CD11/CD18 receptor complex on leukocytes[112] and corresponding receptors on endothelial cells that are of two described types: (1) the endothelial-leukocyte adhesion molecule[53] and intercellular adhesion molecule-1.[113] Expression of receptors on leukocytes is stimulated by *N*-formylmethionyl-leucyl-phenylalanine and PAF. Receptor expression on endothelial cells is stimulated by IL-1. Lipopolysaccharide, TNF, and leukotriene B4 stimulate receptor expression on both leukocytes and endothelial cells.[114]

Evidence has been adduced by Engler et al[115-117] that leukocytes can adhere to endothelium within an ischemic zone of myocardium during both the ischemic and the early reperfusion periods and block more than half of the available capillaries. One adherent leukocyte, trailed by a rouleau of packed red blood cells, tends to occlude each involved capillary. Measured blood flow can progressively decrease during ischemia and reperfusion, particularly in the endocardium, but a considerable percentage of individual capiliaries can become obstructed without increasing total resting vascular resistance or decreasing average flow because of the parallel arrangement of channels. Obstruction of several adjacent capillaries may result in underperfusion of individual cells, however. In an area neighboring the blocked capillaries, mean diffusion distances for oxygen, normally about 1.5 cell diameters, can be increased, and the local partial pressure of oxygen can approach zero. A process of this sort that causes scattered occlusions in the capillary network could expose individual cells to a hypoxic microenvironment but would largely escape detection by the current techniques for measuring blood flow. Granulocytes, labeled with indium 111, have been observed to accumulate as early as 1 hour after a 60-minute period of brain ischemia in an incremental air embolism model in the dog,[118] and the degree of accumulation has shown an

inverse relationship with local blood flow and evoked response recovery.

Neutrophils and macrophages undergo a respiratory burst in which oxygen consumption increases twofold to 20-fold and glucose metabolism via the hexose monophosphate shunt increases.[94] Greater than 90% of the oxygen consumed during this respiratory burst can be accounted for by O^-_2 secretion. The enzyme system responsible for O^-_2 generation has been identified as a membrane-associated nicotinamide-adenine dinucleotide phosphate (NADP[H], NAD[H] oxidase (Fig 4).

Release of granule constituents from leukocytes could potentially extend tissue damage in an ischemic injury zone. Weiss,[119] in a recent review of tissue destruction by neutrophils, has described an intricate interaction between NADPH oxidase-derived oxygen metabolites and granule-based toxins that had been formulated during the course of millions of years of evolution. Superoxide, generated by the neutrophil, is rapidly converted to H_2O_2. The hydrogen peroxide is converted almost quantitatively by the very abundant enzyme, myeloperoxidase (constitutes up to 5% of the dry weight of neutrophils), to hypochlorous acid in the following reaction:

$$H_2O_2 + Cl^- + H^+ \xrightarrow{\text{Myeloperoxidase}} HOCl + H_2O.$$

Hydroxyl radical production by the neutrophil has not been demonstrated. Hypochlorous acid is the sole active ingredient in household liquid bleach, and a million neutrophils can produce a sufficient amount to destroy 150 000 *Escherichia coli* organisms in milliseconds. In a physiologic environment, HOCl reacts with primary or secondary amines to generate a complex family of nitrogen-chlorine (N-Cl) derivatives termed *chloramines*[120]:

$$R'\ RNH + HOCl \rightarrow R'RNCl + H_2O.$$

The question arises as to whether HOCl and chloramines are cytotoxic in vivo. Some clinical data bearing on this question emerge from experience in World War I in which HOCl and chloramines were given in large quantities to wounded soldiers as a local irrigation solution to combat infection. Microbes in the wounds were killed, but the HOCl and chloramine did not seem to cause tissue damage or interfere with wound healing.[121]

Oxidants as a mediator of tissue damage tend to be short lived, react only once, and cannot select targets. Enzymes, stored in neutrophil granules, are long lived, react repeatedly, and can select targets, all of which would seem advantageous. Of more than 20 enzymes in neutrophil granules, three have the greatest potential to mediate tissue destruction. One is a serine proteinase, elastase. The other two are metalloproteinases, collagenase and gelatinase. Despite the destructive potential of proteinases, neutrophil oxidants remain the focus of most of the current literature as final mediators of tissue damage for three reasons. First, there are powerful plasma antiproteinases in vivo that rapidly and irreversibly inhibit the serine proteinases. Second, metalloproteinases are synthesized in latent, inactive forms, and physiologically relevant mechanisms of activation have been heretofore obscure. Third, antioxidants exert strong anti-inflammatory effects in a number of experimental models.

Elastase can degrade almost all components of the extracellular matrix, cleave immunoglobulins and complement and clotting factors, and attack intact cells.[122] It is inhibited in vivo by α-proteinase inhibitor primarily and also by α_2-macroglobulin and secretory leukoproteinase inhibitor. The α_1-proteinase inhibitor forms an irreversible enzyme-inhibitor complex at a rate that approaches the diffusion-controlled limit, so that the half-time of elastase in vivo is about 0.6 milliseconds, and all activity is inhibited in about 3 milliseconds.[123] Chlorinated oxidants destroy the antiproteinase shield. A critical methionine at position 358 on the α_1-proteinase inhibitor becomes oxidized, causing a 2000-fold decrease in the rate of association between neutrophil elastase and the modified α_1-proteinase inhibitor. As a result, the in vivo half-time of elastase increases from 0.6 milliseconds to 1.2 seconds. α_2-macroglobulin and secretory leukoproteinase inhibitor are also oxidatively inactivated. This creates a microenvironment in which elastase can attack host tissues. Neutrophil collagenase and gelatinase degrade various forms of collagen. These enzymes, however, are released in latent, inactive forms,[124] but HOCl can activate them.[125]

It appears, then, that oxidation products, such as HOCl and chloramines, can combine with granule-based proteinases and enable neutrophils to subvert all the intrinsic and host-erected barriers that normally serve to protect host tissue from injury. By inactivation of a series of key proteinase inhibitors and the simultaneous activation of latent proteinases, neutrophils can create an environment in which elastase, collagenase, and gelatinase are able to exert destructive effects more efficiently and with greater specificity than could be achieved by oxidants alone. Although this model is very attractive, and cell damage by extracted granule constituents has been demonstrated in a number of models in vitro (as summarized by Harlan[98]), the role of these enzymes in reperfusion injury has not been directly studied.

Leukocyte accumulation during ischemia and reperfusion has been demonstrated in a variety of organs, and direct and indirect evidence has been adduced by participation of leukocytes in reperfusion injury through rheologic effects, toxic oxygen products, granule constituents and phospholipase products. However, as in adult respiratory distress syndrome where increased alveolar capillary membrane permeability is often attributed to neutrophil mediation, there are several possible relationships between neutrophil accumulation and the development of lung pathology that require critical analysis before accepting a causal role for neutrophils.[126]

Cholesterol

Brief periods of high-cholesterol intake, lasting several days to several weeks and leading to hypercholesterolemia, may cause an acute injury to endothelium through an unidentified mechanism. Such dietary indiscretion might also exacerbate reperfusion injury in the event of myocardial ischemia. Isolated, perfused hearts from rabbits that were fed normal chow that had been supplemented with 2% cholesterol for 2 to 3 weeks were subjected to 30-minute ischemia, followed by 3.5 hours of reperfusion.[127] Vascular permeability (indicated by uptake and clearance of iodine 125-bovine serum albumin) and vascular resistance were increased during reperfusion in these hearts compared with those from animals that were fed normal chow or fed cholesterol-supplemented chow for longer periods (5 to 16 weeks).

Cholesterol supplementation for 2 to 3 weeks was also associated with increased vascular permeability in the absence of ischemia in this study, suggesting that the altered metabolic milieu accompanying an abrupt increase in cholesterol consumption directly injures coronary endothelium. These effects are independent of occlusive coronary artery disease produced by the atherogenic influence of chronic hypercholesterolemia. Indeed, the direct toxicity to the vasculature disappears during protracted states of elevated

cholesterol.

Golino et al[128] have demonstrated that infarct size in acutely hypercholesterolemic, nonatherosclerotic rabbits is roughly double that of normocholesterolemic animals after a standard ischemic injury and that the zones of nonreperfusion were strikingly larger in the former group. In a subsequent work,[129] the same group demonstrated that after feeding rabbits a 2% cholesterol-enriched diet for 3 days, 30 minutes of myocardial ischemia and 5.5 hours of reperfusion led to a more than fourfold greater accumulation of indium 111-labeled platelets in the injury zone and a larger infarct size compared with animals receiving normal chow. Goat anti-rabbit platelet serum decreased platelet counts by about 98% and eliminated the differences between hypercholesterolemic and normocholesterolemic animals with respect to platelet accumulation, infarct size, and extent of reperfusion impairment. The work suggests that the adverse effects of acute hypercholesterolemia on reperfusion injury are platelet dependent. However, monocyte adherence to endothelium has been observed to increase up to 50-fold during acute hypercholesterolemia, raising the possibility that this cell type also participates.[130]

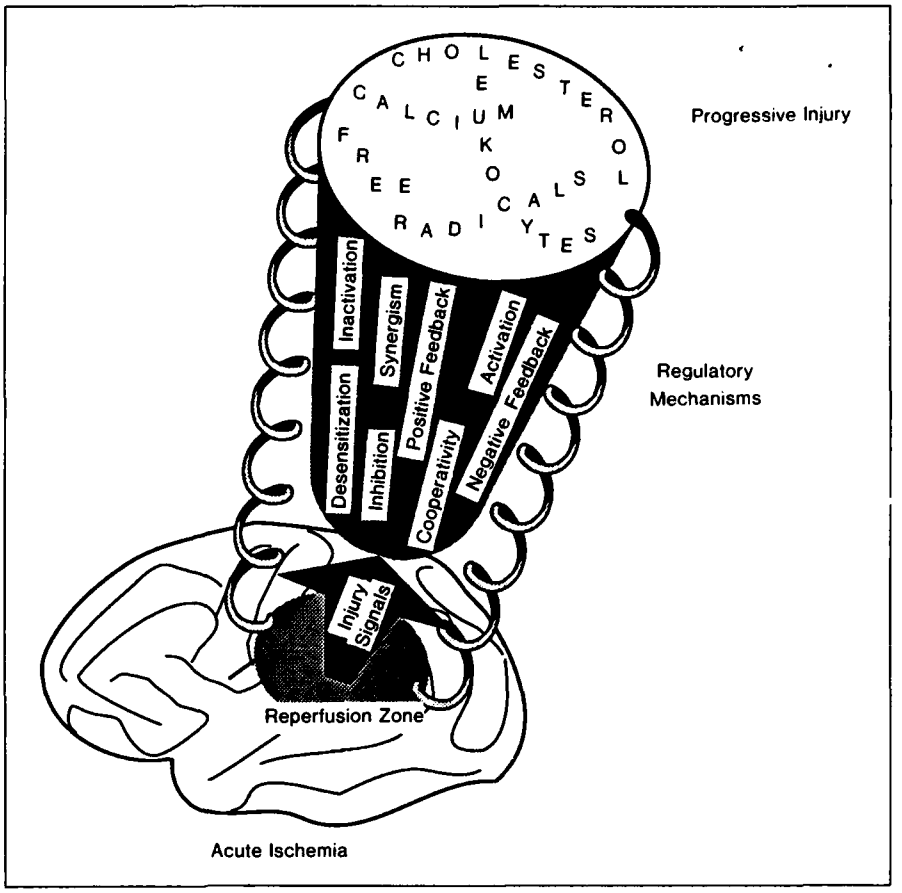

Fig 5.—A schematic overview of reperfusion injury. See text for discussion.

CONCLUSION

As presented in this brief, general review, the postulated mechanisms underlying reperfusion injury are multifactoral and intricately interconnected. They are also governed by positive and negative feedback control, cooperativity, synergism, and maintenance of homeostatic balance by means of counterpoised antagonists. A schematic overview of reperfusion injury is presented in Fig 5, which depicts an interpretation of the data discussed in this review and should be viewed as hypothetical. A core of tissue in the brain exposed to ischemia enters a phase of progressing injury during reperfusion after having been incited by "injury signals," a theoretical construct that could include eicosanoids, PAF, cytokines, transcription factors that regulate gene expression, and conversion of the luminal surface of endothelium from an anticoagulant to a procoagulant membrane. In response, there is a perturbation of the homeostatic balance of many interrelated systems. Multiple mediators are involved, and the loss of regulation sets into motion a cascade of damaging events. The loss of regulation and perturbation of homeostatic balance are represented by a spring that should pull the tissue core back from the progressive injury position but that has been sprung. The major mechanisms, currently ascribed to reperfusion injury, are shown on the face of the tissue core that undergoes progressive injury, with letters intermingled to emphasize that the multitude of intricate and diverse processes encompassed by these mechanisms are tightly interwoven as a "seamless web."[131] Some of these diverse processes would include coagulation, fibrinolysis, eicosanoid and PAF production, cytokine production, rheologic disturbances, granule-based toxin release, adhesion receptor expression on cells, conversion of endothelium to a procoagulant state, platelet accumulation, and, probably, many more. The side of the core of tissue that undergoes progressive injury displays a group of reaction properties that contribute to the complexity of the interlocking events in the progressing injury zone.

In general, therapeutic trials have tended to implicate one or more putative mediators of reperfusion injury as a major cause of the problem and have modified the activity of these mediators. This is the conceptually simple approach, and in many trials, it has produced a small positive effect. Definitive therapy, however, has remained elusive despite these efforts. An alternative, but conceptually more difficult approach that takes account of the full network of effectors and regulators of reperfusion injury as a constellation of minor causes may be required to bring postischemic tissue damage to a minimum. It is possible that the therapeutic problem could be attacked by exploring the regulatory mechanisms that preserve homeostatic balance under normal circumstances and by learning how to curb the putative injury signals (Fig 5).

This review was funded by National Institutes of Health grant 1R01 NS28225-01 and the Naval Medical Research and Development Command Work Unit 61153N MR04101.001-1005.

References

1. Heiss W-D, Rosner G. Functional recovery of cortical neurons as related to degree and duration of ischemia. *Ann Neurol.* 1983;14:294-301.

2. Reimer KA, Jennings RB. The 'wavefront phenomenon' of myocardial ischemic cell death, II: transmural progression of necrosis within the

framework of ischemic bed size (myocardium at risk) and collateral flow. *Lab Invest.* 1979;40:633-644.

3. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? *J Clin Invest.* 1985;76:1713-1719.

4. Weisfeldt ML. Reperfusion and reperfusion injury. *Clin Res.* 1987;35:13-20.

5. Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Pathology.* 1960;70:68-78.

6. Bulkley BH, Hutchins GM. Myocardial consequences of coronary artery bypass graft surgery: the paradox of necrosis in areas of revascularization. *Circulation.* 1977;56:906-913.

7. Greene HL, Weisfeldt ML. Determinants of hypoxic and post-hypoxic myocardial contracture. *Am J Physiol.* 1977;232:H526-H533.

8. Shine KI, Douglas AM. Low calcium reperfusion of ischemic myocardium. *J Mol Cell Cardiol.* 1983;15:251-260.

9. DiBona DR, Powell WJ Jr. Quantitative correlation between cell swelling and necrosis in myocardial ischemia in dogs. *Circ Res.* 1980; 47:653-665.

10. Romson JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation.* 1983; 67:1016-1023.

11. Rowe GT, Manson NH, Caplan M, Hess ML. Hydrogen peroxide and hydroxyl radical mediation of activated leukocyte depression of cardiac sarcoplasmic reticulum. *Circ Res.* 1983;53:584-591.

12. Ames A, Wright RL, Kowada M, et al. Cerebral ischemia, II: the no-reflow phenomenon. *Am J Pathol.* 1968;52:437-447.

13. Leaf A. Regulation of intracellular fluid volume and disease. *Am J Med.* 1970;49:291-295.

14. Fischer EG. Impaired perfusion following cerebrovascular stasis: a review. *Arch Neurol.* 1972;29:361-366.

15. Ginsberg MD, Myers RE. The topography of impaired microvascular perfusion in the primate brain following total circulatory arrest. *Neurology.* 1972;22:998-1011.

16. Hossmann KA, Sato K. Recovery of neuronal function after prolonged cerebral ischemia. *Science.* 1970;168:375-376.

17. Hossmann KA, Kleihues P. Reversibility of ischemic brain damage. *Arch Neurol.* 1973; 29:375-384.

18. Brierley JB, Meldrum BS, Brown AW. The threshold and neuropathology of cerebral 'anoxic-ischemic' cell change. *Arch Neurol.* 1973; 29:367-374.

19. Levy DE, Brierley JB, Plum F. Ischaemic brain damage in the gerbil in the absence of 'no-reflow.' *J Neurol Neurosurg Psychiatry.* 1975; 38:1197-1205.

20. Harrison MJG, Sedal L, Arnold J, Russell RWR. No-reflow phenomenon in the cerebral circulation of the gerbil. *J Neurol Neurosurg Psychiatry.* 1975;38:1190-1193.

21. Halsey JH Jr, Ganji S, Erdmann W, Mardis HM. Studies in cerebral ischemia. *Microvasc Res.* 1977;13:363-370.

22. Hallenbeck JM. Prevention of postischemic impairment of microvascular perfusion. *Neurology.* 1977;27:3-10.

23. Levy DE, van Uitert RL, Pike CL. Delayed postischemic hypoperfusion: a potentially damaging consequence of stroke. *Neurology.* 1979;29:1245-1252.

24. Pulsinelli WA, Levy DE, Duffy TE. Regional cerebral blood flow and glucose metabolism following transient forebrain ischemia. *Ann Neurol.* 1982;11:499-509.

25. Siesjo BK. Cell damage in the brain: a speculative synthesis. *J Cereb Blood Flow Metab.* 1981;1:155-185.

26. Schanne FAX, Kane AB, Young EE, Faber JL. Calcium dependence of toxic cell death: a final common pathway. *Science.* 1979;206:700-702.

27. Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol.* 1982; 11:491-498.

28. Smith M-L, Auer RN, Siesjo BK. The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia. *Acta Neuropathol (Berl).* 1984;64:319-332.

29. Rothman SM, Olney JW. Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann Neurol.* 1986;19:105-111.

30. Novelli A, Reilly JA, Lysko PG, Henneberry RC. Glutamate becomes neurotoxic via the N-methyl D-aspartate receptor when intracellular energy levels are reduced. *Brain Res.* 1988; 451:205-212.

31. DeWood MA, Spores J, Notske R. Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. *N Engl J Med.* 1980;303:897-902.

32. Laffel GL, Braunwald E. Thrombolytic therapy: a new strategy for the treatment of acute myocardial infarction. *N Engl J Med.* 1984; 311:710-717.

33. The thrombolysis in myocardial infarction study group. *N Engl J Med.* 1985;312:932-936.

34. Del Zoppo GJ. Thrombolytic therapy in cerebrovascular disease. *Stroke.* 1988;19:1174-1179.

35. Ryan US, Ryan JW, Crutchley DJ. The pulmonary endothelial surface. *FASEB J.* 1985; 44:2603-2609.

36. Ryan US, Ryan JW. Surface properties of pulmonary endothelial cells. *Ann NY Acad Sci.* 1983;416:441-456.

37. Dietrich WD, Busto R, Ginsberg MD. Cerebral endothelial microvilli: formation following global forebrain ischemia. *J Neuropathol Exp Neurol.* 1984;43:72-83.

38. Clouse LH, Comp PC. The regulation of hemostasis: the protein C system. *N Engl J Med.* 1986;314:1298-1304.

39. Esmon CT. The regulation of natural anticoagulant pathways. *Science.* 1987;235:1348-1352.

40. Bachmann F, Egbert IR, Kruithof KO. Tissue plasminogen activator: chemical and physiological aspects. *Semin Thromb Hemost.* 1984;10:6-17.

41. Verstraete M, Collen D. Thrombolytic therapy in the 80's. *Blood.* 1986;67:1529-1541.

42. Stern D, Nawroth P, Marcum J, et al. Interaction of antithrombin III with bovine aortic segments: role of heparin in binding and enhanced anticoagulant activity. *J Clin Invest.* 1985;75:272-279.

43. Moncada S. Prostacyclin and arterial wall biology. *Arteriosclerosis.* 1982;2:193-207.

44. Crutchley DJ, Ryan US, Ryan JW. Effects of aspirin and dipyridamole on the degradation of adenosine diphosphate by cultured cells derived from bovine pulmonary artery. *J Clin Invest.* 1980;66:29-35.

45. Becker CG, Harpel PC. Alpha 2-macroglobulin on human vascular endothelium. *J Exp Med.* 1976;144:1-9.

46. Rosenblum WI. Biology of disease: aspects of endothelial malfunction and function in cerebral microvessels. *Lab Invest.* 1986;55:252-268.

47. Brox JH, Osterud B, Bjorklid E, Fenton JW II. Production and availability of thromboplastin in endothelial cells: the effects of thrombin, endotoxin and platelets. *Br J Haematol.* 1984; 57:239-246.

48. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA Jr. Interleukin-1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J Exp Med.* 1984;160:618-623.

49. Lyberg T, Galdal KS, Evensen SA, Prydz H. Cellular cooperation in endothelial cell thromboplastin synthesis. *Br J Haematol.* 1983;53:85-95.

50. Libby P, Ordovas JM, Auger KR, Robbins AH, Birinyi LK, Dinarello CA. Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult human vascular endothelial cells. *Am J Pathol.* 1986;124:179-185.

51. Bussolino F, Brevario F, Tetta C, Aglietta M, Mantouani A, Dejana E. Interleukin-1 stimulates platelet-activating factor production in cultured human endothelial cells. *J Clin Invest.* 1986;77:2027-2033.

52. Nawroth PP, Handley DA, Esmon CT, Stern DM. Interleukin-1 induces endothelial cell procoagulant while suppressing cell surface anticoagulant activity. *Proc Natl Acad Sci U S A.* 1986;83:3460-3464.

53. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA Jr. Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes and related leukocyte cell lines. *J Clin Invest.* 1985;76:2003-2011.

54. Bevilacqua MP, Wheeler ME, Pober JS, et al. Endothelial-dependent mechanisms of leukocyte adhesion: regulation by interleukin-1 and tumor necrosis factor. In: Movat HZ, ed. *Leukocyte Emigration and Its Sequelae. Satellite Symposium: Sixth International Congress on Immunology, Toronto, Ontario.* Basel, Switzerland: S. Karger; 1986:79-93.

55. Pober JS, Lapierre LA, Stolpen AH, et al. Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. *J Immunol.* 1987;138:3319-3324.

56. Marks RM, Todd RF III, Ward PA. Rapid induction of neutrophil-endothelial adhesion by endothelial complement fixation. *Nature.* 1989; 339:314-317.

57. Strieter RM, Kunkel SL, Showell HJ, et al. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-α, LPS, and IL-1β. *Science.* 1989;243:1467-1469.

58. Wiggins RC, Loskutoff DJ, Cochrane CJ, Griffin JH, Edgington TS. Activation of rabbit Hageman Factor by homogenates of cultured rabbit endothelial cells. *J Clin Invest.* 1980; 65:197-206.

59. Loesberg C, Gonsalves MD, Zandbergen J, et al. The effect of calcium on the secretion of factor VIII-related antigen by cultured human endothelial cells. *Biochim Biophys Acta.* 1983; 763:160-168.

60. Levine JD, Harlan JM, Harker LA, Joseph ML, Counts RB. Thrombin-mediated release of factor VIII antigen from human umbilical vein endothelial cells in culture. *Blood.* 1982;60:531-534.

61. Ingerman-Wojenski C, Silver MJ, Smith JV, Macarak E. Bovine endothelial cells in culture produce thromboxane as well as prostacyclin. *J Clin Invest.* 1981;67:1292-1296.

62. Piper PJ, Galton SA. Generation of leukotriene B-4 and leukotriene E-4 from porcine pulmonary artery. *Prostaglandins.* 1984;28:905-914.

63. Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science.* 1983;220:568-575.

64. Braquet P, Touqui L, Shen TY, Vargaftig BB. Perspectives in platelet-activating factor research. *Pharmacol Rev.* 1987;39:97-145.

65. McIntyre TM, Zimmerman GA, Satoh K, Prescott SM. Cultured endothelial cells synthesize both platelet-activating factor and prostacyclin in response to histamine, bradykinin, and adenosine triphosphate. *J Clin Invest.* 1985;76:271-280.

66. Camussi G, Aglietta M, Malavasi F, et al. The release of platelet-activating factor from human endothelial cells in culture. *J Immunol.* 1983;131:2397-2403.

67. Dubois C, Bissonnette E, Rola-Pleszczynski M. Platelet-activating factor (PAF) enhances tumor necrosis factor production by alveolar macrophages: prevention by PAF receptor antagonists and lipoxygenase inhibitors. *J Immunol.* 1989;143:964-970.

68. Rodgers GM, Shuman MA. Prothrombin is activated on vascular endothelial cells by Factor Xa and calcium. *Proc Natl Acad Sci U S A.*

1983;80:7001-7005.

69. Stern DM, Drillings M, Kisiel W, Nawroth P, Nossel HL, LaGamma KS. Activation of Factor IX bound to cultured bovine aortic endothelial cells. *Proc Natl Acad Sci U S A.* 1984;81:913-917.

70. Cerveny TJ, Fass DN, Mann KG. Synthesis of coagulation factor V by cultured aortic endothelium. *Blood.* 1984;63:1467-1474.

71. Van Mourik JA, Lawrence DA, Loskutoff DJ. Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. *J Biol Chem.* 1984;259:14914-14921.

72. Juhan-Vague I, Moerman B, De Cock F, Aillaud MF, Collen D. Plasma levels of a specific inhibitor of tissue-type plasminogen activator (and urokinase) in normal and pathological conditions. *Thromb Res.* 1984;33:523-530.

73. Angleton P, Chandler WL, Schmer G. Diurnal variation in tissue plasminogen activator and its rapid inhibitor. *Circulation.* 1987;76(suppl 4):339.

74. Matsubara T, Ziff M. Superoxide anion release by human endothelial cells: synergism between a phorbol ester and a calcium ionophore. *J Cell Physiol.* 1986;127:207-210.

75. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* 1980;288:373-376.

76. Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature.* 1987;327:524-526.

77. Radomski MW, Palmer RMJ, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet.* 1987;2:1057-1058.

78. Yanagisawa M, Kurihara H, Kimura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 1988;332:411-415.

79. Zimmerman ANE, Hulsmann WC. Paradoxical influence of calcium ions on the permeability of cell membranes of the isolated rat heart. *Nature.* 1966;211:646-647.

80. Young W. Ca paradox in neural injury: a hypothesis. *Central Nervous System Trauma.* 1986;3:235-251.

81. Braunwald E. Mechanism of action of calcium-channel-blocking agents. *N Engl J Med.* 1982;307:1618-1627.

82. White BC, Winegar CD, Wilson RF, Hoehner PJ, Trombley JH Jr. Possible role of calcium blockers in cerebral resuscitation: a review of the literature and synthesis for future studies. *Crit Care Med.* 1983;11:202-207.

83. Murphy JG, Marsh JD, Smith TW. The role of calcium in ischemic myocardial injury. *Circulation.* 1987;75(suppl 5):V15-V24.

84. Siesjo BK, Bengtsson F. Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: a unifying hypothesis. *J Cereb Blood Flow Metab.* 1989;9:127-140.

85. Uematsu D, Greenberg JH, Reivich M, Karp A. In vivo measurement of cytosolic free calcium during cerebral ischemia and reperfusion. *Ann Neurol.* 1988;24:420-428.

86. Steenbergen C, Murphy E, Levy L, London RE. Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. *Circ Res.* 1987;60:700-707.

87. Nakayami H, Ginsberg MD, Dietrich WD. (S-emopamil), a novel calcium channel blocker and serotonin S2 antagonist, markedly reduces infarct size following middle cerebral artery occlusion in the rat. *Neurology.* 1988;38:1667-1673.

88. Grotta JC, Pettigrew LC, Rosenbaum D, Reid C, Rhoades H, McCandless D. Efficacy and mechanism of action of a calcium channel blocker after global cerebral ischemia in rats. *Stroke.* 1988;19:447-454.

89. Cheung JY, Bonventre JV, Malis CD, Leaf A. Calcium and ischemic injury. *N Engl J Med.* 1986;314:1670-1676.

90. Del Maestro RF. An approach to free radicals in medicine and biology. *Acta Physiol Scand Suppl.* 1980;492:153-168.

91. Halliwell B, Gudteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J.* 1984;219:1-14.

92. Southorn PA, Powis G. Free radicals in medicine, I: chemical nature and biologic reactions. *Mayo Clin Proc.* 1988;63:381-389.

93. McCord JM. Oxygen-derived radicals: a link between reperfusion injury and inflammation. *FASEB J.* 1987;6:2402-2406.

94. Fantone JC, Ward PA. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *Am J Pathol.* 1982;107:397-418.

95. Kinuta Y, Kimura M, Itokawa Y, Ishikawa M, Kikuchi H. Changes in xanthine oxidase in ischemic rat brain. *J Neurosurg.* 1989;71:417-420.

96. Patt A, Harken AH, Burton LK, et al. Xanthine oxidase-derived hydrogen peroxide contributes to ischemia reperfusion-induced edema in gerbil brains. *J Clin Invest.* 1988;81:1556-1562.

97. Kontos HA. Oxygen radicals in cerebral vascular injury. *Circ Res.* 1985;57:508-516.

98. Harlan JM. Neutrophil-mediated vascular injury. *Acta Med Scand.* 1987;715(suppl):123-129.

99. Schmid-Schonbein GW. Capillary plugging by granulocytes and the no-reflow phenomenon in the microcirculation. *FASEB J.* 1987;46:2397-2401.

100. Engler RL, Schmid-Schonbein GW, Pavelec RS. Leukocyte capillary plugging in myocardial ischemia and reperfusion in the dog. *Am J Pathol.* 1983;111:98-111.

101. Weissmann G, Smolen JE, Korghak HM. Release of inflammatory mediators from stimulated neurophils. *N Engl J Med.* 1980;303:27-34.

102. Moncada S, Higgs EA. Arachidonate metabolism in blood cells and the vessel wall. *Clin Haematol.* 1986;15:273-292.

103. Berenberg JL, Ward PA. Chemotactic factor inactivator in normal human serum. *J Clin Invest.* 1973;52:1200-1206.

104. Wright DG, Gallin JI. A functional differentiation of human neutrophil granules: generation of C5a by a specific (secondary) granule product and inactivation of C5a by azurophil (primary) granule products. *J Immunol.* 1977; 119:1068-1076.

105. Clark RA, Klebanoff SJ. Chemotactic factor inactivation by the myeloperoxidase-hydrogen peroxide-halide system. *J Clin Invest.* 1979; 64:913-920.

106. Blank ML, Lee T, Fitzgerald V, Snyder F. A specific acetylhydrolase for 1-alkyl-2-acetyl-sn-glycero-3- phosphocholine (a hypotensive and platelet-activating lipid). *J Biol Chem.* 1981; 256:175-178.

107. Travis J, Giles PJ, Porcelli L, Reilly CF, Baugh R, Powers J. Human leucocyte elastase and cathepsin G: structural and functional characteristics. *Ciba Found Symp.* 1979;75:51-68.

108. Goldstein IM, Kaplan HB, Edelson HS, Weissmann G. Ceruloplasmin: a scavenger of superoxide anion radicals *J Biol Chem.* 1979; 254:4040-4045.

109. Stroncek DF, Vercellotti GM, Huh PW, Jacob HS. Neutrophil oxidants inactivate alpha-1 protease inhibitor and promote PMN-mediated detachment of cultured endothelium: protection by free methionine. *Arteriosclerosis.* 1986;6:332-340.

110. Seeger W, Suttorp N, Schmidt F, Neuhof H. The glutathione redox cycle as a defense system against hydrogen-peroxide-induced prostanoid formation and vasoconstriction in rabbit lungs. *Am Rev Respir Dis.* 1986;133:1029-1036.

111. Harlan JN, Levine JD, Callahan KS, Schwartz BR. Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. *J Clin Invest.* 1984;73:706-713.

112. Smith CW, Rothlein R, Hughes BJ, et al. Recognition of an endothelial determinant for CD 18-dependent human neutrophil adherence and transendothelial migration. *J Clin Invest.* 1988; 82:1746-1756.

113. Dustin ML, Staunton DE, Springer TA. Supergene families meet in the immune system. *Immunol Today.* 1988;9:213-215.

114. Harlan JM, Schwartz BR, Wallis WJ, Pohlman TH. The role of neutrophil membrane proteins in neutrophil emigration. In: Movat HZ, ed. *Leukocyte Emigration and Its Sequelae, Satellite Symposium: Sixth International Congress on Immunology, Toronto, Ontario.* Basel, Switzerland: S. Karger; 1986:94-104.

115. Engler RL, Dahlgren MD, Morris DD, Peterson MA, Schmid-Schönbein GW. Role of leukocytes in response to acute myocardial ischemia and reflow in dogs. *Am J Physiol.* 1986;251(Heart Circ Physiol 20):H314-H322.

116. Engler RL, Dahlgren MD, Peterson MA, Dobbs A, Schmid-Schönbein GW. Accumulation of polymorphonuclear leukocytes during 3-h experimental myocardial ischemia. *Am J Physiol.* 1986;251(Heart Circ Physiol 20):H93-H100.

117. Engler R. Consequences of activation and adenosine-mediated inhibition of granulocytes during myocardial ischemia. *FASEB J.* 1987; 46:2407-2412.

118. Hallenbeck JM, Dutka AJ, Tanishima T, et al. Polymorphonuclear leukocyte accumulation in brain regions with low blood flow during the early postischemic period. *Stroke.* 1986;17:246-253.

119. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med.* 1989;320:365-376.

120. Test ST, Lampert MB, Ossanna PJ, Thoene JG, Weiss SJ. Generation of nitrogen-chlorine oxidants by human phagocytes. *J Clin Invest.* 1984;74:1341-1349.

121. Smith JL, Drennan AM, Rettie T, Campbell W. Experimental observations on the antiseptic action of hypochlorous acid and its application to wound treatment. *BMJ.* 1915;2:129-136.

122. Janoff A. Elastase in tissue injury. *Ann Rev Med.* 1985;36:207-216.

123. Travis J, Salvesen GS. Human plasma proteinase inhibitors. *Annu Rev Biochem.* 1983; 52:655-709.

124. Weiss SJ, Peppin GJ. Collagenolytic metalloenzymes of the human neutrophil: characteristics, regulation and potential function in vivo. *Biochem Pharmacol.* 1986;35:3189-3197.

125. Weiss SJ, Peppin G, Ortiz X, Ragsdale C, Test ST. Oxidative autoactivation of latent collagenase by human neutrophils. *Science.* 1985; 227:747-749.

126. Glauser FL, Fairman RP. The uncertain role of the neutrophil in increased permeability pulmonary edema. *Chest.* 1985;88:601-607.

127. Tilton RG, Cole PA, Zions JD, et al. Increased ischemia-reperfusion injury to the heart associated with short-term diet-induced hypercholesterolemia in rabbits. *Circ Res.* 1987;60:551-559.

128. Golino P, Maroko PR, Carew TE. The effect of acute hypercholesterolemia on myocardial infarct size and the no-reflow phenomenon during coronary occlusion-reperfusion. *Circulation.* 1987;75:292-298.

129. Golino P, Maroko PR, Carew TE. Efficacy of platelet depletion in counteracting the detrimental effect of acute hypercholesterolemia on infarct size and the no-reflow phenomenon in rabbits undergoing coronary artery occlusion-reperfusion. *Circulation.* 1987;76:173-180.

130. Joris I, Zand T, Nunnari JJ, Krolikowski FJ, Majno G. Studies on the pathogenesis of atherosclerosis, I: adhesion and emigration of mononuclear cells in the aorta of hypercholesterolemic rats. *Am J Pathol.* 1983;113:341-358.

131. Ratnoff OD. Some relationships among hemostasis, fibrinolytic phenomena, immunity, and the inflammatory response. *Adv Immunol.* 1969;10:145-227.

132. Siesjo BK. Historical overview: calcium, ischemia, and death of brain cells. *Ann NY Acad Sci.* 1988;522:638-661.

Printed and Published in the United States of America